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Aneuploidy is a significant factor in the tumorogenic progression of breast cells. Failure in cell cycle checkpoint controls causes aneuploidy. Our goal has been to characterise checkpoint proteins required to maintain the fidelity of chromosome segregation. The anaphase inhibitor Pds1p is critically involved in this regulation. We identified a novel Pds1-dependent checkpoint pathway that prevents aneuploidy by coordinating DNA replication with mitotic anaphase. It is a distinct control system from the established S-phase checkpoint pathways previously described. A detailed characterisation of this checkpoint pathway is underway. Two more genes required for regulated chromosome segregation were identified, Rad23 and Ddi1. Structure/function studies revealed a likely mechanism through which Rad23 and Ddi1 may regulate Pds1 (by binding to ubiqutinated Pds1). Rad23 and Ddi1 contain a novel protein interaction domain (UBA) that binds to ubiquitin and ubiquitinated proteins. Rad23 and Ddi1 UBAs are essential for genetic interactions with a *pds1* mutant. These genes have closely related human homologues that are likely to be required for human checkpoint controls. Failure in such checkpoint mechanisms are a potential cause of aneuploidy that contributes to the etiology of breast cancer.

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Dan Clark 6/22/99

PI - Signature Date

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6.20.99

Statement Of Work
(Revised for the period 6/1/1998-5/31/2000)

Isolation of genes required for the regulated separation of sister chromatids

PI: Duncan J. Clarke, Ph.D.
DAMD17-97-1-7059

Technical objective 1 (revised and expanded)
Screens for new proteins involved in chromatid segregation

Task 1. Months 13-36. Detailed analysis of the Pds1-dependent checkpoint that couples DNA replication with mitosis.

Task 2. Months 13-24. Determine the relationship between the sensitivity of *pds1* mutant cells to the DNA replication inhibitor hydroxyurea and the *pds1* mutant S-phase checkpoint defect. Does the checkpoint defect result in chromosome non-disjunction?

Task 3. Months 13-36. Compare the Pds1-dependent S-phase checkpoint pathway with the established checkpoint pathways that couple DNA replication with mitosis.

Task 4. Months 13-24. Analysis of other factors that affect Pds1 function.

Task 5. Months 13-36. Determine the functions of the *pds1* mutant suppressors, Rad23 and Ddi1.

Technical objective 2. Characterization of CST1
(Discontinued - no longer relevant; see 1998 annual report)

Technical objective 3. Characterization of DAM1
(Discontinued - no longer relevant; see 1998 annual report)

Introduction

Subject, purpose and scope of research

Aneuploidy is a prevalent genetic affliction and a significant factor in malignancies including those of the breast. Since aneuploidy can result from mitotic non-disjunction, understanding the process of chromosome segregation is crucial. Segregation fidelity relies on elaborate mechanics, precisely regulated by cell cycle checkpoint controls. The purpose of this research has been to identify new proteins that are required for this regulation. In budding yeast, chromosome segregation is controlled by ubiquitin-dependent degradation of the anaphase inhibitor Pds1p. During an unpurturbed cell cycle, Pds1p becomes poly-ubiquitinated at the metaphase to anaphase transition by multi-enzyme APC/cyclosome complexes; the modified forms are then recognized and degraded by 26S proteasome particles. Pds1p degradation initiates displacement of chromatid cohesion proteins, thus allowing sister separation and the onset of anaphase. Our goal has been to characterize new proteins that function to maintain the fidelity of chromosome segregation. Pds1p is critically involved in this regulation. Therefore we aimed to identify new proteins that interact with Pds1p. We describe the identification of two genes which are required for regulated chromosome segregation: these genes have very closely related human homologues. In addition we have identified a novel Pds1-dependent checkpoint pathway that is crucially required to prevent aneuploidy. A detailed characterisation of this checkpoint pathway is underway.

Body of 1999 annual report

1. Results

In the 1998 annual report we described evidence that Pds1 is required for S-phase checkpoint control. In the last 12 months we have performed a detailed analysis of this checkpoint system. This study has revealed that a novel Pds1p-dependent checkpoint coordinates DNA replication with mitotic anaphase. The Pds1-dependent checkpoint is a distinct control system from the established S-phase checkpoint pathways previously described. Thus, there are 2 separate pathways involved in coordinating replication with anaphase. Loss of either of these checkpoint controls causes aneuploidy.

We have also made progress in characterizing the functions of Rad23 and Ddi1, 2 novel high copy suppressors of a *pds1* mutant (*pds1-128*). Rad23 and Ddi1 are likely to be important regulators of chromosome segregation. However, dissection of their functions in this process is a complex task. Rad23 is a multi-domain protein required for nucleotide excision repair (NER), but has a second unknown function responsible for the suppression of the *pds1* S-phase checkpoint defect. Ddi1 has a very similar structure to Rad23, and thus may perform similar functions. The multi-functionality of these proteins makes analysis of the phenotypes of *rad23* and *ddi1* mutants inherrently complicated. For this reason we decided to employ a structure/function study of Rad23 and Ddi1. First we asked whether specific domains of Rad23 and Ddi1 are required for high copy suppression of *pds1* mutant cells. This lead to a very exciting observation - that the UBA domains of Rad23 and Ddi1 are essential for the suppression. The function of UBA domains is not known, though they are present in different classes of enzyme involved in ubiquitin-dependent proteolysis; an intriguing coincidence given the dependence of Pds1p proteolysis on the ubiquitin system. Futher analysis of the UBAs revealed that they are novel protein interaction domains. Rad23 and Ddi1 form hetero- and homo-dimers and in addition can interact with ubiquitin and ubiquitinated proteins. We find that most of these interactions are UBA-dependent. The UBA-dependent suppression of the *pds1* S-phase checkpoint defect suggests that the UBAs mediate checkpoint activation.

1.1 Characterization of the Pds1-dependent S-phase checkpoint

1.1a

Premature spindle elongation and loss of sister chromatid cohesion during S-phase in pds1 muntants

For a detailed study of the role of Pds1p in S phase checkpoint control, we

monitored the onset of anaphase in kinetic cell cycle analysis experiments. In budding yeast, mitotic spindles assemble during S-phase. Replication is normally completed before short G2 spindles form, but in the presence of 100mM hydroxyurea (HU), replication proceeds at a reduced rate and the S-phase checkpoint must delay anaphase to allow the completion of replication. Under these conditions, loss of S phase checkpoint control can be unequivocally demonstrated by measuring the relative timing of DNA replication and the onset of anaphase. Wild type and *pds1-128* cells were synchronized in G1, then released into rich medium containing 100mM HU. To estimate the timing of anaphase onset, sister centromere separation (SCS) was monitored (Fig. 1). Although both strains budded and progressed through S phase with similar timing, SCS was advanced in *pds1-128*. At least 36% of budded *pds1-128* cells had undergone sister centromere separation at a time when most cells were still in S phase, according to FACScan analysis (not shown). Thus, *pds1-128* cells engage in premature sister centromere separation when the coupling of S phase and mitosis is challenged. In addition, mitotic spindles elongated prematurely in the *pds1-128* mutant (Fig. 2). *pds1-128* cells initiated anaphase when about 2/3 of the genome had been replicated.

1.1b

The pds1 mutant S-phase checkpoint defect results in aneuploidy

Uncoupling S phase from mitosis in *pds1-128* cells resulted in aberrant anaphase (Fig 3) that caused aneuploidy. After one generation in the presence of HU, a population of cells with less than 1N DNA content was detectable by FACScan analysis. 30% of the newly divided cells exhibited gain or loss of the centromere region of chromosome IV (Fig. 4), indicating that many nuclei failed to segregate evenly. After 3 generation times in HU, 50% of cells had an excess of centromere region IV signals, and 21% had <1N DNA content. These abortive attempts at anaphase closely resemble those described for *scc1* mutants, in which sister chromatid cohesion fails to become established during S phase. We conclude that *pds1* mutants are defective in S-phase checkpoint control and that this defect results in aneuploidy.

1.1c

The Pds1-dependent checkpoint is a novel S-phase checkpoint pathway

To gain more information about the Pds1-dependent S-phase checkpoint, we compared the checkpoint defect of *pds1-128* with that of other S-phase checkpoint mutants, *mec1* and *rad53*. When the *mec1* checkpoint defect was analysed during partial inhibition of replication, we observed that *mec1* cells initiated anaphase as soon as

spindle assembly occurred (Fig. 5). This was also true of *rad53* mutant cells (not shown). In contrast, although *pds1-128* cells began anaphase prematurely, there was a distinct period in early S phase when mitosis was restrained. Therefore, Pds1, as well as Mec1 and Rad53, is essential to coordinate ongoing DNA replication with mitosis. Although all of these proteins are required, they must act at different times in S phase and in distinct pathways. Mec1/Rad53 must function either independently of Pds1 throughout S phase, or independently of Pds1 in early S phase but upstream of Pds1 later in S phase. We have proposed that an event intrinsic to the progression of DNA replication elicits a switch in the mode of checkpoint regulation (Fig 6). Since Pds1p is required for maintaining sister centromere cohesion, the Pds1p-dependent pathway may operate only once centromere cohesion has been established.

1.2 Structure/function analysis of Rad23 and Ddi1

1.2a

Rad23 UBA2 is dispensible for nucleotide excision repair

We tested whether the C-terminal UBA of Rad23 (UBA2) is required for the nucleotide excision repair function Rad23 by replacing the endogenous *RAD23* gene with a *rad23ΔUBA2[MYC]6X* fusion or a full length *RAD23[MYC]6X* fusion. Both fusion proteins were present in equal amounts in yeast cells (not shown). These strains were wild type with regard to UV sensitivity; clearly Rad23 UBA2 is dispensible for nucleotide excision repair.

1.1b

RAD23 and DDI1 UBA domains are required for suppression of the pds1-128 S-phase checkpoint defect

Rad23p and Ddi1p may have a redundant function in the S phase checkpoint. Since they share a conserved UBA domain, we suspected that these domains might mediate the checkpoint signal. If correct, deletions of the *RAD23* and *DDI1* UBA domains should abolish their suppressor effects on *pds1-128*. Therefore we made truncated versions of *RAD23* and *DDI1* (*GAL1:rad23ΔUBA2* and *GAL1:ddi1ΔUBA*) which lack the C-terminal UBA domains (about 10% of each protein deleted) under the control of the *GAL1* promoter. These were unable to rescue the temperature sensitivity of *pds1-128* when expressed from the *GAL1* promoter, though full length versions of either protein did rescue (Fig 7). The lack of rescue was not due to a reduced stability of the truncated proteins since tagged versions (*GAL1:rad23ΔUBA2[MYC]6X,*

GAL1:RAD23[MYC]6X, *GAL1:ddi1ΔUBA[HIS]6X* and *GAL1:DDI1[HIS]6X*) were present at the same level in yeast cells and gave the same result in the *pds1-128* rescue assay.

The ability of the mutant forms to rescue the hydroxyurea sensitivity of *pds1-128* was also examined. Induction of *GAL1:ddi1ΔUBA* was unable to rescue the hydroxyurea sensitivity (Fig. 8). *GAL1:rad23ΔUBA2* could partially rescue the hydroxyurea sensitivity, consistent with the presence of a second UBA domain located internally (retained in the *GAL1:rad23ΔUBA2* mutant) which may mediate a partial functional interaction with *pds1-128*. Therefore, the UBA domain of Ddi1p is essential for suppression of the *pds1-128* hydroxyurea sensitivity, and deletion of 1 of 2 UBA domains from Rad23 reduces the ability of Rad23 to rescue the hydroxyurea sensitivity *pds1-128* cells.

1.1c

RAD23 and DDI1 UBA domains are required for the enhancement of the esp1 mutant phenotype

To establish the relevance of UBA-dependent suppression of *pds1* by high dosage *RAD23/DDI1*, we investigated possible genetic interactions between *GAL1:rad23ΔUBA2*/*GAL1:ddi1ΔUBA* and *esp1* mutants. The biological relevance of the *ESP1/PDS1* genetic interaction is that Pds1p binds to Esp1p and thereby inhibits the anaphase-promoting activity of Esp1p. We described in the 1998 annual report that *RAD23/DDI1* overexpression enhances the temperature sensitivity of *esp1* muntants. If the UBAs of Rad23/Ddi1 are required for specifically regulating Pds1, the genetic interaction with *esp1* should be UBA-dependent. Indeed, the lethality of strains with a temperature sensitive *esp1* allele was greatly enhanced by over-expression of *RAD23/DDI1* but not by overexpression of *rad23ΔUBA2* or *ddi1ΔUBA* (Fig 9).

2. Summary and Discussion

We are performing a detailed analysis of a novel Pds1p-dependent checkpoint system that coordinates DNA replication with mitotic anaphase. The checkpoint is a distinct control system from the established Mec1/Rad53 pathway. Failure of the Pds1p-dependent checkpoint pathway results in aneuploidy. The genetic and cell biology techniques developed during this work will be important for future analysis of checkpoint controls.

A structure/function analysis of *pds1-128* dosage suppressors (Rad23 and Ddi1) has been initiated. Since Pds1-dependent checkpoint control depends on regulating Pds1

stability, it is logical that Rad23/Ddi1 increase stability of Pds1. Rad23 was recently shown to interact physically with the complex responsible for Pds1 degradation: the M-phase specific 26S proteasome, but this complex apparently functions in DNA repair rather than in protein degradation. It is therefore likely to be significant that we find interactions between Rad23/Ddi1 and ubiquitin and ubiquitinated proteins. Moreover, these interactions are dependent on the Rad23/Ddi1 UBA domains, and as described above, these domains are required for suppression of *pds1*. This provides an alternative mechanism through which Rad23/Ddi1 may stabilise Pds1. The human homologues of *RAD23* and *DDI1* are structurally conserved, suggesting that they have analogous functions in human cell cycle control. In terms of training, the structure/function studies have been a valuable learning experience. Currently, the analysis has been extended to a random and directed mutagenesis of the UBA domains and we are collaborating to solve crystal structures containing the relevant interaction partners.

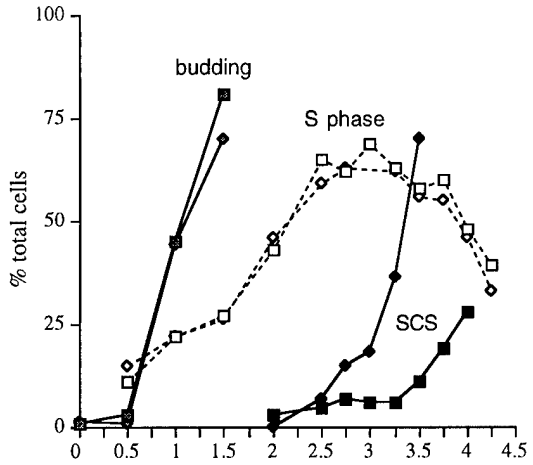


Fig 1

Loss of sister centromere cohesion during S phase in *pds1-128* cells
G1-arrested wild type and *pds1-128* cells were released into rich medium containing 100mM HU. Budding index and sister separation at the centromeric region of chromosome IV (visualized by utilizing the binding of tetR-GFP fusion proteins to tandemly integrated tetO sequences at the centromere-linked *TRP1* locus) were scored, and S phase-index estimated from FACScan analysis. Wild type, squares; *pds1-128*, diamonds; gray fill, budding index; open symbols, S phase cells; solid fill, separation of sister centromeres.

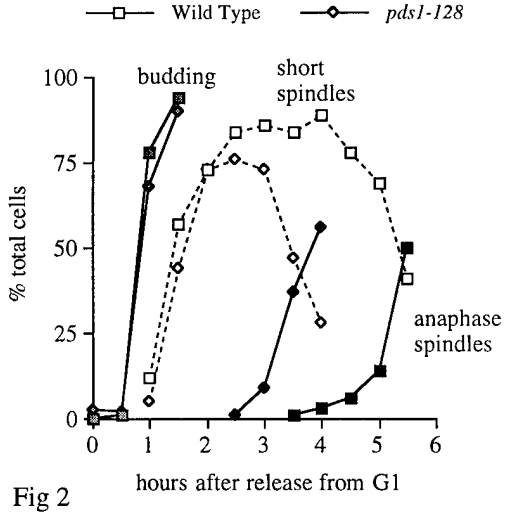


Fig 2

Premature spindle elongation during S phase in *pds1-128* cells. Experimental design as in Fig 1. Budding index (gray fill), short spindle formation (open symbols) and spindle elongation (solid fill) were scored. Spindles were visualized by expressing a *GFP-TUB1* construct.

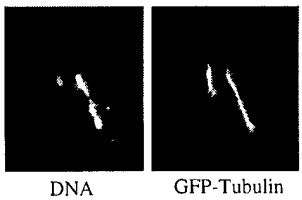


Fig 3

Aberrant mitosis part-way through S phase in *pds1-128* cells. Aberrant late anaphase; spindle fully elongated, nuclei not divided/unequally divided.

Fig 4

Aneuploidy induced by growth with 100mM hydroxyurea in *pds1-128*. The cell depicted has an undivided nucleus away from bud neck with 2 GFP signals. Chromosome IV centromere detected by tetR-GFP fusions binding to tetO sequences at the TRP1 locus.

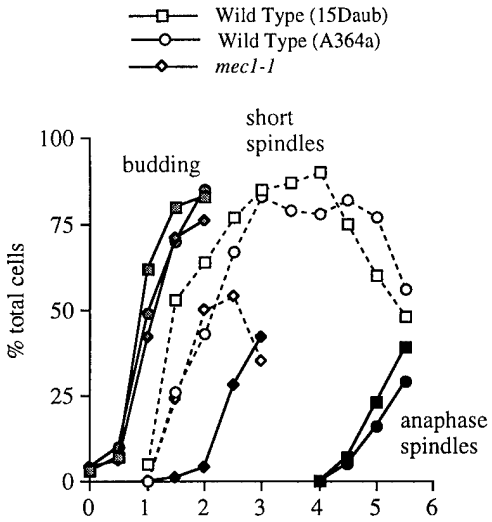


Fig 5

Early S phase spindle elongation in *mec1-1* cells. Experimental design as in Fig 1. Budding index (gray fill), short spindle formation (open symbols) and spindle elongation (solid fill) were scored. Spindles were visualized by expressing a *GFP-TUB1* construct.

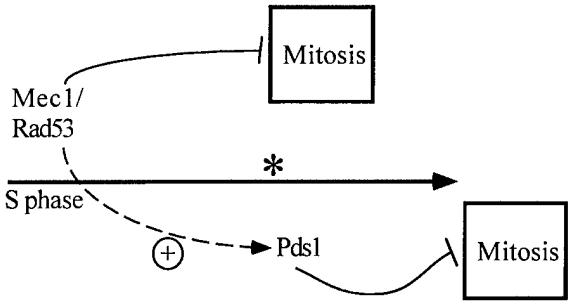


Fig 6

Model for coupling replication with mitosis. Possible modes of S phase checkpoint regulation: Mec1/Rad53 and Pds1 are essential components of distinct and sequential checkpoint pathways which block mitosis, one active in early S phase, the other active part-way through S phase, either operating in parallel (solid arrows) or operating in series (additional broken arrow). Part-way through S phase there is a switch (*) in the mode of checkpoint control.

Fig7

UBA-dependent suppression of *pds1-128*. Rad23 and Ddi1 UBA are required for supression of *pds1-128* temperature sensitivity. Full length or truncated versions of *RAD23* and *DDI1* expressed from the GAL1 promotor.

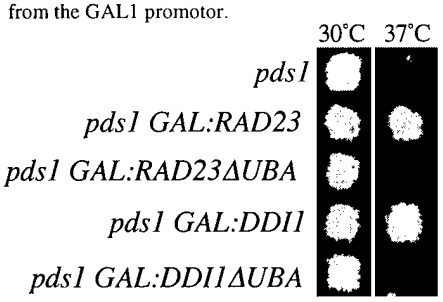


Fig8

UBA-dependent suppression of *pds1-128* HU sensitivity. Rad23 and Ddi1 UBA are required for supression of *pds1-128* HU sensitivity. Full length or truncated versions of *RAD23* and *DDI1* expressed from the GAL1 promotor. *GAL1:RAD23ΔUBA* partially suppressed the HU sensitivity (not shown).

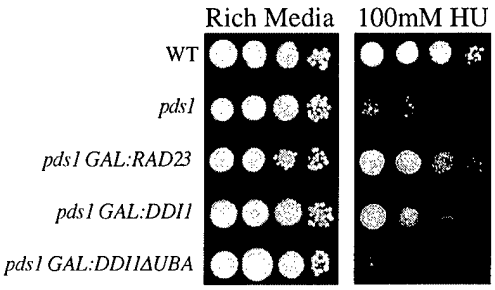
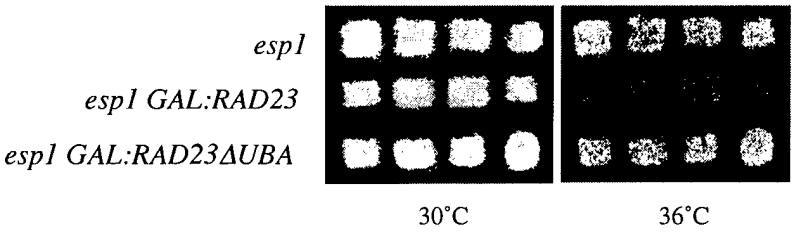


Fig 9

UBA-dependent enhancement of *esp1*. Rad23 and Ddi1 UBA are required for enhancement of *esp1* temperature sensitivity. Full length or truncated versions of *RAD23* and *DDI1* expressed from the GAL1 promotor. Enhancement was also UBA-dependent in the case of DDI1 (not shown).



Key Research Accomplishments

- Identification of a novel Pds1-dependent checkpoint control that couples DNA replication with mitosis
- Characterization of Pds1, Mec1 and Rad53 functions in S-phase checkpoint control
- Identification of high copy suppressors of a *pds1* mutant (Rad23 and Ddi1)
- Demonstration that Rad23 and Ddi1 UBAs are required for suppression of *pds1*
- Demonstration that Rad23 and Ddi1 UBAs are novel protein interaction domains
- Demonstration that Rad23 and Ddi1 bind to ubiquitin

Reportable Outcomes

Manuscripts

1. Clarke, D.J., Segal, M., Mondésert, G. and Reed, S.I. (1999). **The Pds1 anaphase inhibitor and Mec1 kinase define distinct checkpoints coupling S phase with mitosis in budding yeast.** *Current Biol.* 9:365-368.
2. Clarke, D.J., and Giménez-Abián, J.F. (1999). **Invited Review: Checkpoints controlling entry into mitosis**. *Bioessays*, In preparation.
3. Clarke, D.J., Mondésert, G., Segal, M., and Reed, S.I. **Dosage supressors of pds1 checkpoint control defects**. In preparation.
4. Bertolaet, B., Clarke, D.J., and Reed, S.I. **UBA domain interactions in budding yeast**. In preparation.
5. Clarke, D.J., Segal, M., Jensen, S. and Reed, S.I. **Anatomy of the S-phase checkpoint in budding yeast**. In preparation.

Platform Presentations

1. **Upstream elements activating the Pds1-dependent anaphase-checkpoint in yeast.** Duncan J. Clarke, Marisa Segal, Guillaume Mondésert and Steven I. Reed. *EMBO Fellows Meeting, 12-14th July 1998, Heidelberg, Germany.*
2. **Distinct and sequential S-phase checkpoint controls in yeast.** Duncan J. Clarke, Marisa Segal, Sanne Jensen, Guillaume Mondésert and Steven I. Reed. *The Salk Institute Inaugural Cell Cycle Meeting, 18th-22nd June 1999, La Jolla, CA.*
3. **Cyclin-dependent kinase and Cks/Suc1 interact with the proteasome in yeast to control proteolysis of M-phase targets.** Mark H. Watson, Peter Kaiser, Vincent Moncollin, Duncan J. Clarke, Bonnie L. Bertolaet, Steven I. Reed and Eric Bailly. *The Salk Institute Inaugural Cell Cycle Meeting, 18th-22nd June 1999, La Jolla, CA.*

Poster Presentations

1. **Role Of Pds1 In S-Phase Checkpoint Control.** Duncan J. Clarke, Marisa Segal, Guillaume Mondésert and Steven I. Reed. *British Society for Cell Biology Spring Meeting 1999, Manchester, U.K.*

Submitted Applications for Funding

1. NIH Grant (RO1).

The Pds1 anaphase inhibitor and Mec1 kinase define distinct checkpoints coupling S phase with mitosis in budding yeast

Duncan J. Clarke*†, Marisa Segal*†, Guillaume Mondésert‡ and Steven I. Reed*

In most eukaryotic cells, DNA replication is confined to S phase of the cell cycle [1]. During this interval, S-phase checkpoint controls restrain mitosis until replication is complete [2]. In budding yeast, the anaphase inhibitor Pds1p has been associated with the checkpoint arrest of mitosis when DNA is damaged or when mitotic spindles have formed aberrantly [3,4], but not when DNA replication is blocked with hydroxyurea (HU). Previous studies have implicated the protein kinase Mec1p in S-phase checkpoint control [5]. Unlike *mec1* mutants, *pds1* mutants efficiently inhibit anaphase when replication is blocked. This does not, however, exclude an essential S-phase checkpoint function of Pds1 beyond the early S-phase arrest point of a HU block. Here, we show that Pds1p is an essential component of a previously unsuspected checkpoint control system that couples the completion of S phase with mitosis. Further, the S-phase checkpoint comprises at least two distinct pathways. A Mec1p-dependent pathway operates early in S phase, but a Pds1p-dependent pathway becomes essential part way through S phase.

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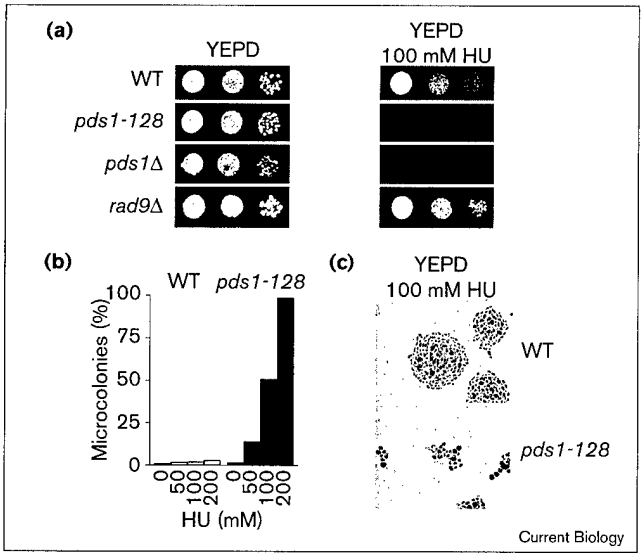
Results and discussion

In budding yeast, initiation of anaphase is controlled by the ubiquitin-dependent degradation of the anaphase inhibitor Pds1p. This process constitutes a target of late cycle checkpoint controls [6]. To address whether Pds1p is required for S-phase checkpoint control, we adopted two approaches. First, we used a hypomorphic *pds1* allele, *pds1-128*, that causes a less severe temperature sensitivity than a null allele and is, therefore, more amenable to the study of cell-cycle events in synchronous populations. Although the restrictive temperature for growth of *pds1-128* cells is 37°C, DNA damage and spindle assembly checkpoint defects are apparent at 26°C, comparable to those previously described for the *pds1-1* mutant [3,4]. Although a replication block induced by 400 mM HU caused *pds1-128* and *pds1Δ* cells to checkpoint-arrest (data not shown), these mutants were highly sensitive to non-replication-arresting doses of HU (50–100 mM; Figure 1a). On solid medium containing 100 mM HU, *pds1-128* mutants formed microcolonies (Figure 1b,c). In liquid medium containing 100 mM HU, at least 50% of *pds1-128* cells lost viability per generation (see Supplementary material published with this article on the internet). Crucially, *rad9Δ* cells, defective for DNA damage checkpoint control, were not sensitive to 100 mM HU (Figure 1a). Hence, the sensitivity of *pds1* mutants does not result from a Rad9p-dependent DNA damage checkpoint defect.

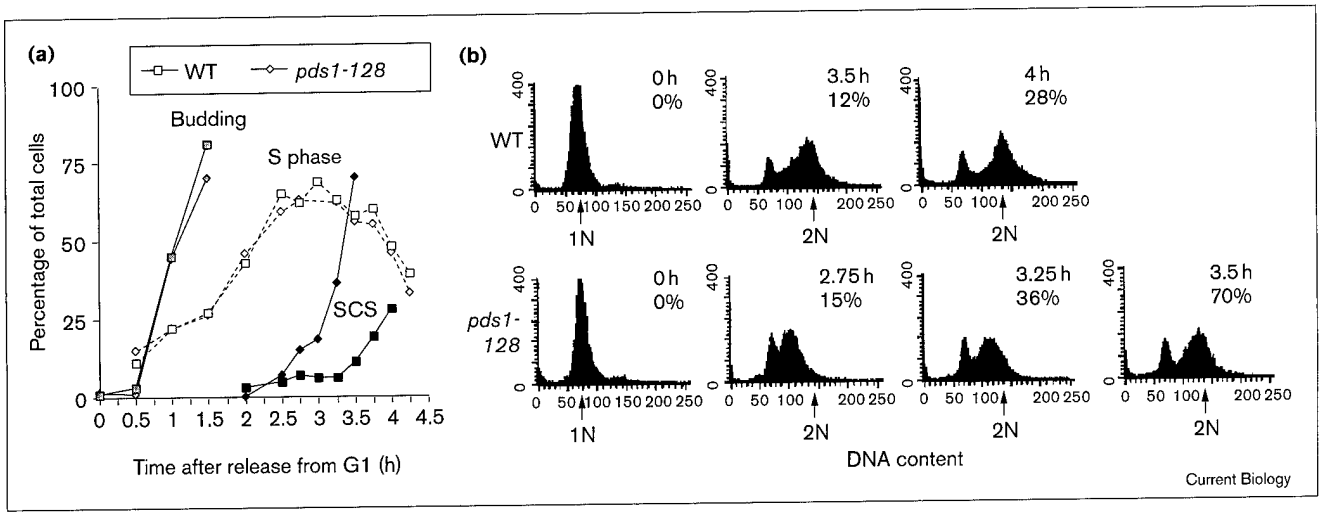
Second, we performed kinetic studies in which the coupling of S phase with mitosis was challenged by partial inhibition of replication. Normally, replication is completed before short G2 spindles form [7]. In the presence of 100 mM HU, replication proceeds at a reduced rate [7]. Only once cells have budded and formed short mitotic spindles must anaphase be delayed to allow the completion of replication. Under these conditions, loss of S-phase

Figure 1



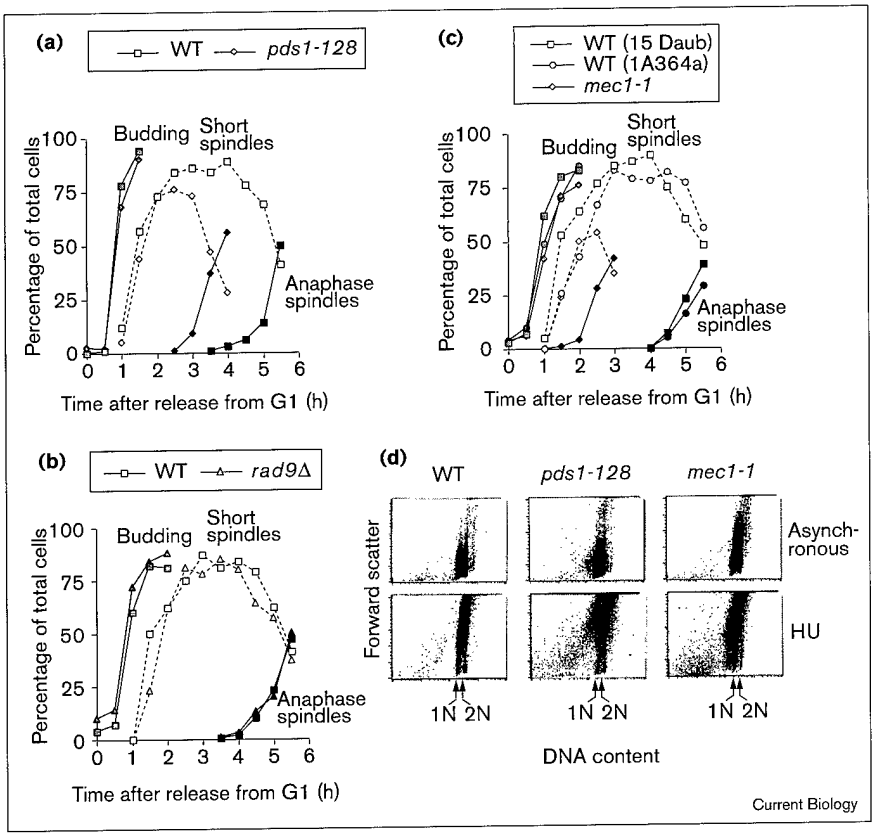
Sensitivity of *pds1* mutants to HU. Serial dilutions of mid-log cells from wild type (WT), *pds1* or *rad9* mutants were spotted onto solid YEPD medium or onto YEPD containing HU and grown at 30°C (25°C for *pds1Δ*). **(a)** Spot growth was recorded after 2–3 days. After 24 h, microcolonies were **(b)** counted and **(c)** photographed.

Figure 2



Loss of sister centromere cohesion during S phase in *pds1-128* cells. Wild-type (WT) and *pds1-128* cells were arrested in G1 with 200 ng/ml α factor (at least 90% unbudded cells), then released into rich medium containing 100 mM HU at 30°C. **(a)** Cell aliquots were taken at given time intervals for scoring budding index (gray symbols), sister centromere separation (SCS) for chromosome IV (black symbols), the percentage of cells in S phase (open symbols), and for FACScan analysis of DNA content. **(b)** Histograms show the DNA content of cells in samples at selected time points (the time following α factor release and the percentage of separated centromeres are indicated above the corresponding graph). When *pds1-128* cells divided before DNA replication was complete, nuclear division was unequal. This resulted in G1 cells with greater than 1N DNA content; the next S phase further increased the DNA content of these cells, a fact apparent late in this time course (notice the sub-2N peak for the *pds1-128* cells at 3.5 h).

Figure 3



Distinct checkpoint control defects in *pds1* and *mec1* mutants. Strains were G1-arrested as in Figure 2, then released into rich medium containing 100 mM HU at 26°C. Cell aliquots were taken at given time intervals to score budding index (gray symbols), short spindle formation (open symbols) and spindle elongation (black symbols), and for FACScan analysis of DNA content. Each strain replicated DNA with similar kinetics (the kinetics of replication in *mec1-1* cells could not be determined because these cells began anaphase before much DNA had been replicated; data not shown). **(a)** Wild-type (WT) and *pds1-128* cells. **(b)** Wild-type and *rad9Δ* cells. **(c)** Wild-type (in both the 15Daub and A364a genetic backgrounds) and *mec1-1* (A364a genetic background) cells. **(d)** Dot plots of DNA content versus forward scatter for wild-type, *pds1-128* and *mec1-1* cells grown in rich media with or without 100 mM HU for three generations following release from α factor. The positions of 1N and 2N DNA content are indicated. In the presence of 100 mM HU, both *pds1-128* and *mec1-1* cultures contain populations of cells with less than 1N DNA content: about 10% of the total cells after the first division (7 h after release from α factor) of both mutants; 21% for *pds1-128* after three generations (14 h after release); and 31% for *mec1-1* after three generations (14 h after release).

checkpoint control can be unequivocally demonstrated by measuring the relative timing of budding, DNA replication, spindle assembly and the onset of anaphase. Others have identified proteins required for checkpoint arrest when replication has been blocked [5]; we examined checkpoint control during ongoing DNA replication.

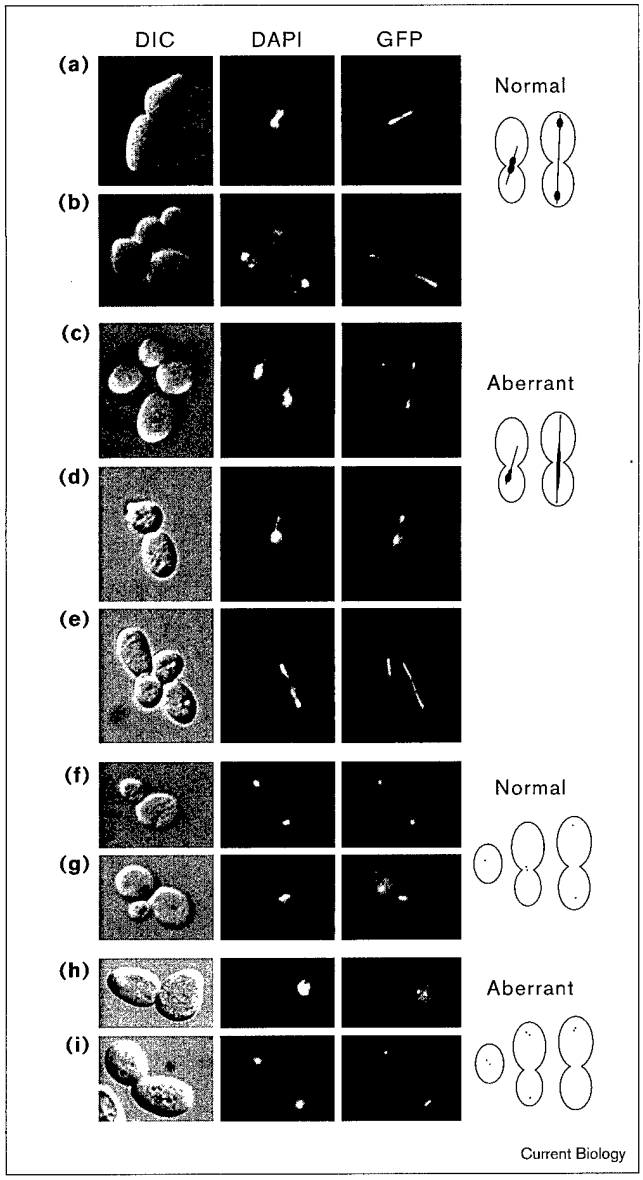
Wild-type and *pds1-128* cells were synchronized in G1 by adding α factor, then released into liquid YEPD medium containing 100 mM HU. To estimate the timing of the onset of anaphase, sister centromere separation was monitored (Figure 2a). Although both strains budded and progressed through S phase with similar timing, sister centromere separation was advanced in *pds1-128* cells. At least 36% of budded *pds1-128* cells had undergone sister centromere separation at a time when most cells were still in S phase, according to FACScan analysis (Figure 2b). Thus, *pds1-128* cells engage in premature sister centromere separation when the coupling of S phase and mitosis is challenged.

Other aspects of anaphase also occurred prematurely in the *pds1-128* mutants. For example, *pds1-128* cells elongated mitotic spindles about 2 hours before wild-type cells (Figure 3a), even though both strains replicated DNA with similar timing (data not shown). Considering the relatively short G2 interval in budding yeast, the 2 hour advancement of spindle elongation indicates that most *pds1-128* cells must have initiated anaphase before replication was complete. Indeed, FACScan profiles (data not shown, but see Figure 1) revealed that most cells had less than a 2N DNA content at a time when the bulk of the population had initiated spindle elongation. Wild-type and *pds1-128* cells progressed through S phase and began anaphase with indistinguishable timing in the absence of HU (see Supplementary material).

Uncoupling S phase from mitosis in *pds1-128* cells had several consequences. Following release from α factor in the presence of 100 mM HU, 50% of *pds1-128* cells engaged in an aberrant mitosis (Figure 4). After the first division, a population of cells with less than 1N DNA content was detectable by FACScan analysis (Figure 3d), and 30% of the newly divided cells exhibited gain or loss of the centromere region of chromosome IV (17% of cells had no centromere region IV signal, 13% had a signal >1; Figure 4), indicating that many nuclei failed to segregate evenly. After three generation times in HU, 50% of cells had an excess of centromere region IV signals and 21% had <1N DNA content. These abortive attempts at anaphase closely resemble those described for *scc1* mutants, in which sister chromatid cohesion fails to become established during S phase [8].

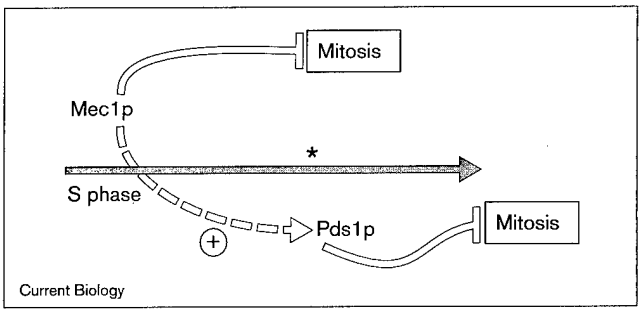
The sensitivity of *pds1* mutants to HU could partly reflect the DNA damage checkpoint defect of these cells because,

Figure 4



Aberrant mitosis part way through S phase in *pds1-128* cells. **(a,b,f,g)** Wild-type and **(c–e,h,i)** *pds1-128* cells grown in medium containing 100 mM HU. (a–e) Images are from differential interference contrast (DIC) microscopy, 4,6-diamidino-2-phenylindole (DAPI) staining to visualize the chromosomes, and fluorescence microscopy to visualize spindles (using GFP-labeled TUB1). (f–i) Images are from DIC microscopy, DAPI staining and fluorescence microscopy to visualize the centrosome of chromosome IV (using GFP-labeled tetR). (a) Normal early anaphase: spindle partly elongated, nucleus stretched at bud neck. (b) Normal late anaphase: spindle fully elongated, nucleus divided. (c,d) Aberrant early anaphases: spindles partly elongated, nuclei abnormally positioned and stretched. (e) Aberrant late anaphase: spindle fully elongated, nucleus not divided. (f) Normal late anaphase: nuclei divided, each has one GFP signal. (g) Normal early anaphase: nucleus at bud neck, slightly separated centromeres (adjacent GFP signals). (h) Aneuploid *pds1-128* cell: undivided nucleus away from bud neck; two GFP signals. (i) Aneuploid late anaphase *pds1-128* cells; one nucleus has two GFP signals.

Figure 5



Model for coupling replication with mitosis. Possible modes of S-phase checkpoint regulation: Mec1p and Pds1p are essential components of distinct and sequential checkpoint pathways that block mitosis, one active in early S phase, the other active part way through S phase, either operating in parallel (solid lines) or operating in series (additional broken arrow). Part way through S phase (gray arrow), there is a switch (*) in the mode of checkpoint control.

during S phase, HU inevitably causes replicative stress. The severe aneuploidy induced by HU treatment in *pds1* mutants suggests, however, that loss of coordination between replication and mitosis is responsible for the lethality. Indeed, *rad9Δ* cells, defective for all known aspects of DNA damage checkpoint control [9] but not sensitive to HU (Figure 1), were proficient at coupling S phase with mitosis, using the same experimental conditions as described above (Figure 3b). Therefore, the S-phase Pds1p-dependent checkpoint system is Rad9p-independent.

We have shown that Pds1p is required to couple ongoing replication with mitosis. An outstanding issue is why *pds1* mutants arrest efficiently when replication is completely blocked. One intriguing explanation could be that S phase and mitosis are coupled by distinct checkpoint pathways that act sequentially. To test this, we compared the checkpoint defect of *pds1-128* with that of *mec1*, the prototypic S-phase checkpoint mutant [5]. When the *mec1* checkpoint defect was analysed during partial inhibition of replication, we observed that *mec1* cells initiated anaphase as soon as spindle assembly occurred (Figure 3c). In contrast, although *pds1-128* cells began anaphase prematurely, there was a distinct period in early S phase when mitosis was restrained. This was also true of the *pds1* null mutant (see Supplementary material).

Rather than regulating mitotic kinase activity, the budding yeast DNA damage and spindle assembly checkpoints inhibit anaphase directly by stabilizing the inhibitor Pds1p [6]. This work demonstrates that Pds1p, as well as Mec1p, is essential to coordinate ongoing DNA replication with mitosis. Although both of these proteins are required, they must act at different times in S phase and in distinct pathways (Figure 5). Mec1p must function either independently of Pds1p throughout S phase, or independently of Pds1p in early S phase but upstream of Pds1p later in S phase. We propose that an event intrinsic to the progression of DNA replication elicits a switch in the mode of checkpoint regulation. As Pds1p is required for maintaining sister centromere cohesion, the Pds1p-dependent pathway may operate only once centromere cohesion has been established, an event that is likely to be completed by mid-S phase, when centromeric regions have been replicated [8,10]. Alternatively, Pds1p may be required following the initiation of late replication origins, because Mec1p was recently shown to be essential for an early S-phase checkpoint that inhibits late origin firing [11]. In response to DNA damage and spindle assembly checkpoint controls, Pds1p degradation is inhibited. By analogy, ongoing DNA replication may signal Pds1p stabilization in order to restrain anaphase until replication is completed.

Supplementary material

Additional methodological details, yeast strain genotypes and supplementary figures are published with this article on the internet.

Acknowledgements

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ROLE OF PDS1 IN S PHASE CHECKPOINT CONTROL. DUNCAN J. CLARKE, MARISA SEGAL, GUILLAUME MONDÉSERT AND STEVEN I. REED.
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Most eukaryotic cells confine DNA replication to S phase of the cell cycle. During this interval, S phase checkpoint controls restrain mitosis until replication is complete. Other checkpoint controls respond to DNA damage or mitotic spindle defects. In budding yeast, the anaphase inhibitor Pds1p has been associated with checkpoint arrest of mitosis when DNA is damaged or if mitotic spindles have formed aberrantly, but not when DNA replication is blocked with hydroxyurea. Previous studies implicate the protein kinase Mec1p in S phase checkpoint control. Unlike *mec1*, *pds1* mutants efficiently inhibit anaphase when replication is blocked. However, this does not exclude an essential S phase checkpoint function of Pds1 beyond the early S phase arrest point of a hydroxyurea block. We find that Pds1p is an essential component of a previously unsuspected checkpoint control system that couples the completion of S phase with mitosis. Further, the S phase checkpoint comprises at least 2 distinct pathways. A Mec1p-dependent pathway operates early in S phase, but part-way through S phase, a Pds1p-dependent pathway becomes essential.

DISTINCT AND SEQUENTIAL S-PHASE CHECKPOINT CONTROLS IN YEAST.

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Most eukaryotic cells confine DNA replication to S-phase of the cell cycle. During this interval, checkpoint controls restrain mitosis until replication is complete. In budding yeast, S-phase checkpoint defects have been revealed by kinetic studies in which the relative timing of DNA replication and anaphase are measured. Using GFP-Tubulin to visualize mitotic spindles, and a GFP-tagged centromere to measure sister centromere separation, we demonstrated that the S phase checkpoint comprises 2 pathways[1]. In early S-phase, a Mec1p-dependent pathway operates; part-way through S-phase, a Pds1p-dependent pathway becomes essential. These checkpoint systems are distinct, but moreover, operate sequentially. Thus necessitating an event intrinsic to the progression of DNA replication that elicits a switch in the mode of checkpoint regulation. Since Pds1p is required for maintaining sister chromatid cohesion, the Pds1p-dependent pathway may operate only once cohesion has been established at specific chromosomal sites such as centromeres. Alternatively, the initiation of late replication origins may precipitate the checkpoint switch, since Mec1p is essential for an early S-phase checkpoint that inhibits late origin firing. These issues are currently being addressed.

We have also investigated possible roles of other checkpoint proteins in S-phase control. Rad9p, Chk1p and Mad2p are not required, dismissing possible interplay between the DNA damage or spindle assembly checkpoint pathways with the late S-phase checkpoint pathway. Mec1 and Rad53 have long been known to function in S-phase checkpoint control. However, we find that *mec1* and *rad53* mutants display strikingly different phenotypes in relation to the function of Pds1p. These analyses have revealed how the S-phase checkpoint pathways are organized in yeast.

[1] Clarke DJ, Segal M, Mondésert G and Reed SI. (1999). The Pds1 anaphase inhibitor and Mec1 kinase define distinct checkpoints coupling S-phase with mitosis in budding yeast. *Curr. Biol.* 9:365-368

CYCLIN-DEPENDENT KINASE AND CKS1 INTERACT WITH THE PROTEASOME TO CONTROL PROTEOLYSIS OF M-PHASE TARGETS IN YEAST.

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Cell cycle specific proteolysis is critical for proper execution of mitosis in all eukaryotes. Ubiquitination and subsequent proteolysis of the mitotic regulators Clb2 and Pds1 depend on the cyclosome/APC and the 26S proteasome. We report here that components of the cell cycle machinery in yeast, specifically the cell cycle regulatory cyclin-dependent kinase Cdc28 and a conserved associated protein Cks1/Suc1, interact genetically, physically, and functionally with components of the 26S proteasome. A mutation in Cdc28 (*cdc28-1N*) that interferes with Cks1 binding, or inactivation of Cks1 itself, confers stabilization of Clb2, the principal mitotic B-type cyclin in budding yeast. Surprisingly, Clb2 ubiquitination *in vivo* and *in vitro* is not affected by mutations in *cks1*, indicating that Cks1 is not essential for cyclosome/APC activity. However, mutant Cks1 proteins no longer physically interact with the proteasome, suggesting that Cks1 is required for some aspect of proteasome function during M-phase specific proteolysis. We further provide evidence that Cks1 function is required for degradation of the anaphase inbibitor Pds1. Stabilization of Pds1 is partially responsible for the metaphase arrest phenotype of *cks1* mutants since deletion of *PDS1* partially releaves the metaphase block in these mutants.

UPSTREAM ELEMENTS ACTIVATING THE PDS1-DEPENDENT ANAPHASE-CHECKPOINT IN YEAST

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In response to DNA damage or incompletely replicated chromosomes, cells activate checkpoint controls which slow or arrest replication and prevent mitosis from being initiated prematurely. Therefore, checkpoints avoid replication of damaged template DNA in S phase and prevent aberrant segregation of damaged chromosomes in mitosis. One mode of checkpoint execution involves preventing the onset of anaphase. During an unperturbed cell cycle in budding yeast, the anaphase inhibitor Pds1p becomes poly-ubiquitinated by multi-enzyme APC/cyclosome complexes; the modified forms are recognized and degraded by 26S proteasome particles: These events initiate the onset of anaphase. Checkpoint arrest appears to be mediated through stabilization of Pds1p; thus, *PDS1* mutants are checkpoint deficient.

Although Pds1p is a key component of the anaphase-checkpoint machinery in yeast, how Pds1p stability is regulated is not known. To identify regulators of Pds1p, a genetic screen was carried out. We have found two proteins which act upstream of Pds1p in the checkpoint pathway (encoded by *PSR1* and *PSR2*; for Pds1p Stability Regulator). Overexpression of *PSR* genes rescues the temperature sensitivity and the checkpoint defect of *PDS1* mutants. Conversely, *PSR* mutants are checkpoint defective. As expected, overexpression of *PSR1* and *PSR2* stabilizes wild type Pds1p.